Impact of short- and long-range forces on protein conformation and adsorption kinetics

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We have studied the adsorption kinetics of the protein amylase at solid/liquid interfaces. Offering substrates with tailored properties, we are able to separate the impact of short- and long-range interactions. By means of a colloidal Monte Carlo approach including conformational changes of the adsorbed proteins induced by density fluctuations, we develop a scenario that is consistent with the experimentally observed three-step kinetics on specific substrates. Our observations show that not only the surface chemistry determines the properties of an adsorbed protein layer but also the van der Waals contributions of a composite substrate may lead to non-negligible effects.

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Introduction

The adsorption of proteins at interfaces plays a crucial role in determining the function of many biological systems, and hence it is the focus of research activities in chemical and medical applications. Adsorption is a thermodynamic process that occurs spontaneously whenever protein-containing aqueous solutions contact solid surfaces, and results in a modification of the sorbent surface and often that of the protein as well [1]. The structure of a protein is closely connected to its function and efficacy, therefore protein adsorption and surface-induced conformational changes are important issues in biocompatibility of materials and have been subject to numerous studies (cf. [2, 3, 4, 5] and references therein). The adsorption of proteins from aqueous solutions is driven by short- and long-range interactions. The main players in the latter are van der Waals and Coulomb contributions. Due to the strong screening of the Coulomb interactions, the question arises what actually is the leading contribution of these two. It is the aim of this paper to study the role of the long-range potential forces and possible conformational changes for the complex process of protein adsorption [6, 7].

Studies of *in-situ* biofilm formation are an experimental and theoretical challenge. The experimental technique must provide a sub-nm spatial resolution in normal sample direction as well as a time resolution in the range of seconds. Additionally, the sample is not accessible directly, rather it is immersed in a liquid and only minute amounts of material can be analyzed. These constraints rule out many common thin film characterization techniques. Surface plasmon resonance spectroscopy (SPR) or the frequency shift of a quartz crystal microbalance upon material adsorption will provide a high sensitivity, yet both methods suffer from a constraint concerning the substrate material. Therefore, ellipsometry seemed to us to be the method of choice for the *in-situ* studies. From the viewpoint of modelling, the atomistic simulation of a complex biofilm with at least hundreds of mutual interacting macromolecules, each of which with its own complexity, is not possible with state-of-the-art computers. Even taking into account the fast development of computational power, the time scale of the adsorption process, which is of the order of minutes, will not be accessible in the next few decades. Therefore, the only route to achieve a comprehensive description is to use a largely simplified protein model that has a level of sophistication in accordance with the experimental characteristics.

In this study, we combine an experimental and a modelling approach, each of which is not able to offer a complete picture of the protein adsorption kinetics. In the ellipsometry studies a systematic variation of the potential forces is provided by using tailored composite substrates. The theoretical investigations are performed by means of Monte Carlo (MC) simulations utilizing an effective particle model. The special focus of the MC investigation is on the relation between conformational changes of the (model) proteins and the adsorption kinetics. By the combination of both methods, we have been able to devise a consistent scenario for the biofilm adsorption. Our findings underline the importance of long-range forces as well as of induced conformational changes of the proteins.

Experiments

The protein under investigation, α-amylase from human saliva, has particular relevance in dental research due to its function in the primary colonization of bacteria leading to plaque formation [8]. As substrate a composite material was used. Silicon wafers with natural (2 nm) and thick (192 nm) silicon oxide surfaces cause identi-

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cal short-range interactions with the adsorbate, since the chemical composition of the surface is identical [9, 10]. However, comparing the long-range van der Waals forces acting between adsorbate and oxide layer with those acting between adsorbate and bulk material through the oxide layer, the strength and sign of the Hamaker constant can be different [9]. Varying the oxide layer thickness enables the strength of the van der Waals forces to be tuned, whilst maintaining all other parameters (protein and salt concentration, temperature, surface composition) constant. This concept has been successfully applied to describe the stability of coatings [9] and has been recently extended to a comprehensive understanding of gecko adhesion [10]. An additional hydrophobization of the oxide surfaces by a self-assembled monolayer of silane molecules allows for a variation of the short-range forces, while maintaining the long-range forces essentially constant. The reason for this is that the strength of the van der Waals forces between an adsorbate and a layer are proportional to the volume of the layer [11].

Analysis of the kinetics facilitates characterization of protein adsorption without the inherent assumptions often associated with the interpretation of isotherms [6]. Optical methods allow for high sampling frequency (in contrast to e.g. scattering techniques), high resolution of the adsorbed amount, and *in-situ* monitoring without alteration of the protein structure or hydrodynamic conditions. Protein adsorption was followed *in-situ* with an imaging ellipsometer (Nanofilm EP^3, Germany) operating via the nulling ellipsometry principle at a wavelength of 532 nm [12]. Samples were mounted in a teflon fluid cell which enabled precise temperature and flow control at two angles of incidence, 65° and 70°. Modeling of the ellipsometric data assumes a homogeneous layer approximation, with de Feijter's method [13] used to determine the adsorbed mass as both thickness and refractive index of such thin transparent films cannot be unambiguously determined by single wavelength ellipsometry [12]. Assuming that the refractive index of a protein in solution is a linear function of its concentration, the absolute amount Γ of adsorbed protein can be determined by $\Gamma = d_f \frac{(n_f - n_a)}{dn/dc}$, where d_f and n_f are the thickness and refractive index of the adsorbed film respectively, n_a is the refractive index of the ambient, and dn/dc is the refractive index increment of the molecules, which was fixed at 0.183 cm^3/g for our measurements [13, 14]. The standard deviation is approximately 0.1 mg/m^2. Both wafer types (2 nm and 192 nm SiO_2) were hydrophilic and were alternatively covered by a monolayer of silanes (octadecyl-trichlorosilane), rendering them hydrophobic [15, 16]. This resulted in four different types of composite substrates with pairwise identical short- or long-range forces, respectively [9, 10]. The layer structure of all surfaces was characterized accurately by *ex-situ* ellipsometry, the rms roughness of all surfaces was below 0.2 nm as determined by scanning probe microscopy of an area of 1 μm^2. α-amylase from human saliva (Fluka no. 10092) was dissolved in a 10 mM phosphate buffer solution at pH 7.0, stored at 4 °C, and used for up to 4 days after preparation. Upon the fluid cell reaching thermal equilibrium, protein was injected under constant flow conditions (Rheodyne Manual Sample Injector), which were maintained throughout a given experiment.

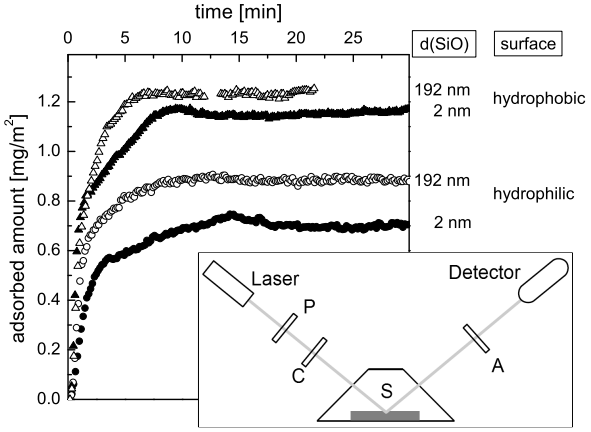

FIG. 1: Adsorption of α-amylase on the four substrates under investigation. Inset: Ellipsometric setup in the PCSA configuration (P: Polarizer, C: Compensator, S: Sample, A: Analyser).

Figure 1 shows the adsorbed amount of α-amylase on the four substrates. As expected from literature [2], we find a higher adsorbed amount on the hydrophobic surfaces. The adsorption on the thick silicon oxide samples (open symbols) exhibits a commonly observed kinetics with a continuously decreasing adsorption rate up to a limiting value of the surface excess. The situation is completely different on the native wafer series (closed symbols). The initial adsorption rate is consistent with that of the thermally grown oxide samples. However, one observes a linear growth (constant growth rate) regime with a defined beginning and end. This occurs on both the hydrophobic and hydrophilic surfaces. The fact that one observes different adsorption kinetic curves on chemically identical surfaces indicates that protein adsorption kinetics are significantly influenced by long-range forces. Additional experiments with various silicon oxide layer thicknesses revealed that a three-step kinetics can be observed for SiO_2 layer thicknesses below 20 nm.

The colloidal approach: Model and Simulation results

In order to explore the microscopic origin of the three-step kinetics we perform MC simulations using a colloidal representation of the protein molecules as spherical particles. The substrate and the solvent are likewise treated as continuous media. Particle-particle as well as particle-surface interactions are described in the framework of the DLVO-theory [17, 18], considering steric repulsion, van der Waals and electrical double layer interactions.

Hamaker's results [19] are used to calculate the van der Waals forces. Approximate expressions for the electrostatic interactions can be obtained using the linear superposition approximation (LSA) [20] together with Sader's equation [21] for the effective far field potential. Almost the complete set of model parameters, e.g. protein net charge and Debye length, are experimentally accessible. Therefore we have chosen parameter values that are consistent with experimental findings rather than model parameters optimizing the agreement between experimental and theoretical results of the adsorption kinetics. In Fig.

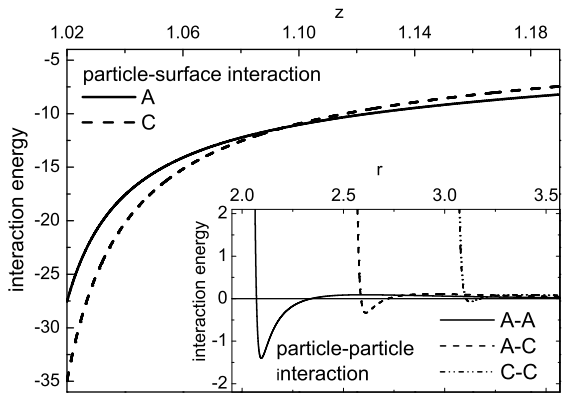

FIG. 2: Conformation dependent particle-surface and particle-particle (inset) potentials. z denotes the distance between the center of a particle and the substrate surface; r is the center-to-center distance of two interacting particles. Energies are given in units of k_BT, z and r in units of the radius of the spheres in conformation A.

2, the solid curves represent the resulting particle-surface and (in the inset) particle-particle potentials.

The single particle MC scheme applied here describes the particle dynamics as a stochastic motion in real and configuration space, similar to the Brownian dynamics method. For this reason we expect to obtain qualitatively the same behavior for the adsorption kinetics. However, the advantage of MC is that internal degrees of freedom of the particles can easily be considered, enabling the complex adsorption behavior of proteins to be modeled.

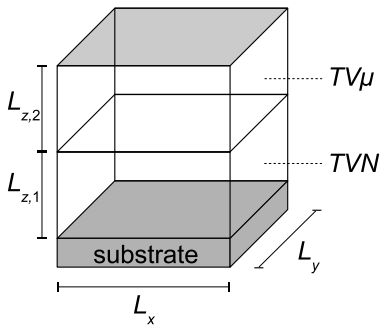

FIG. 3: Sketch of the simulation box.

In order to investigate the time evolution of the surface coverage theoretically it is necessary to choose a simulation volume that is in accordance with the experimental situation. We have therefore divided the simulation volume into two parts (see Fig. 3) [22]. In the upper box a grandcanonical ensemble ($TV\mu$) is applied. In contrast, no particle exchange with an external reservoir is considered in the lower box adjacent to the substrate. Particles can diffuse from the upper box into the lower one and vice versa. In the lower box they are influenced by the attractive substrate such that there is a net particle flux from the upper box into the lower one, until a stationary state is reached. This setup is motivated by the experimental situation where the concentration of proteins in bulk solution remains approximately constant during the adsorption experiment. We use periodic boundary conditions in the x- and y-direction and reflecting boundaries at the top and the bottom of the simulation box. The total height of the simulation box is chosen such that a further increase of $L_{z,1}$ or $L_{z,2}$ does not change the simulation results qualitatively .

Using this basic model we observe the irreversible adsorption of a particle monolayer consistent with the experimental findings. As expected [22], we obtain a conventional shape of the adsorption kinetics characterised by a gradual reduction of the adsorption rate. The robustness of this result indicates that the model has to be extended in order to reproduce the observed three-step kinetics. In the experiments the long-range forces originating from the substrate turn out to change the adsorption kinetics qualitatively. A straightforward physical interpretation of this observation is that the long-range interactions influence the orientation of the proteins in solution, and consequently their initial conformation in the irreversible adsorption process. This physical picture implies, that depending on the nature of the long-range forces, different initial conformations of the adsorbed proteins are possible.

Modeling conformational changes

As there is experimental and theoretical evidence that proteins in the adsorbed state can undergo conformational changes [5, 24, 25] it is crucial for the adsorption kinetics whether the conformation of adsorbed proteins is stable. In order to maintain the computational performance, we extended our model by introducing an internal degree of freedom, which models the different protein conformations. Specifically, particles may adopt three different states: in bulk solution they take on the native (compact) conformation A, if adsorbed to the substrate they either adopt a marginally altered (native-like) conformation B or a substantially altered (denatured) conformation C. Conformational changes $B \to C$ are modeled to be reversible in order to account for the process of partial refolding upon denaturation ($A \to B \to C \to B$) [24, 25]. Trial probabilities are introduced for the thermally activated transitions $B \leftrightarrow C$. As the effective

radius of conformation B is assumed to be of the order of the native conformation A, we choose $B \equiv A$ for simplicity. The denaturation upon adsorption is associated with a larger contact area between the protein molecule and the substrate (spreading), thereby enlarging the binding energy of the proteins, cf. Fig. 2. In the framework of the colloidal approach a conformational change of a protein molecule upon adsorption is represented by a volume conserving deformation of the particle. As a result the effective size of the particle ("particle interaction radius") with respect to its interaction with the substrate is increased while it is reduced with respect to the particle-particle interactions. In order to maintain the computational efficiency it is favorable to keep the spherical geometry of the particles. Consequently, the denaturation is implemented by adapting the particle interaction radii separately for the different contributions to the potentials (see the inset of Fig. 2). Note that particles in conformation C now cover a larger surface area of the substrate. Such a modeling of conformational changes is in the spirit of the Equivalent Sphere Approach (ESA) for the interaction of non-spherical colloidal particles [26]. The transition from compact to extended protein conformations takes place only close to the surface of the substrate. Therefore transitions $A \to C$ are restricted to particles that are in close proximity to the substrate, i.e. for $z \leq 1.1$, where conformation C is energetically favored (see Fig. 2). In contrast, conformational changes $C \to A$ are allowed in the entire simulation volume. This ensures that unbound particles are in the native conformation A. Note that within the model presented a conformational change is indistinguishable from a reorientation of a protein molecule at the surface (top-on $\leftrightarrow$ side-on).

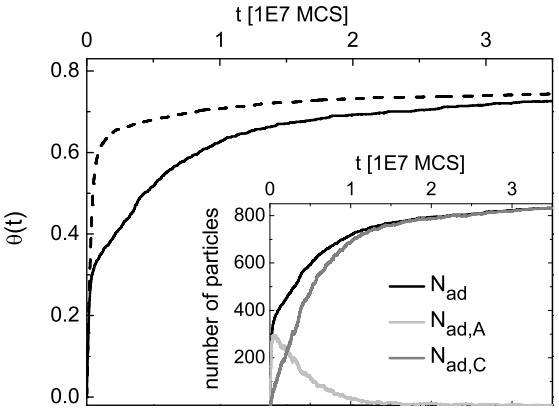

FIG. 4: Adsorption kinetics for particles with (solid line) and without (dashed line) internal degree of freedom. Time t is measured in MC sweeps. Inset: Number of adsorbed particles depending on conformation.

Figure 4 shows the time evolution of the surface coverage $\theta(t) = \frac{\pi}{L_x L_y} N_{ad}(t)$, which is proportional to the number of adsorbed particles per surface area (for definition of $L_{x,y}$ cf. Fig. 3) and corresponds to the experimentally observed quantity adsorbed amount Γ. Compared to the reference curve (without conformational changes), the adsorption kinetics is characterized by an intermediate region with a moderate adsorption rate. In qualitative agreement with the experimental results three regimes of the adsorption kinetics can be distinguished: During the first part of the kinetics the number $N_{ad,C}$ of adsorbed particles in conformation C grows almost as fast as the total number N_{ad} of adsorbed particles (see inset of Fig. 4), because at low surface coverages the particles transform immediately after adsorption to the energetically favored conformation C (see Fig. 5(a),(b)). With increasing surface coverage, the particle-particle interactions become more relevant and induce the growth of A-domains at the surface (see Fig. 5(c),(d)). For these high surface coverages the optimization of particle-particle interactions due to the formation of A-domains overcompensates the unfavorable surface-particle interaction of conformation A. The third step of the adsorption kinetics can be viewed as the ordering transition of a 2d monodisperse system (see Fig. 5(e),(f)). This process leads to a rather slow saturation of the adsorption kinetics compared to the experimental observations. However, for the real system a rearrangement to a closest packed structure is not expected anyway, since at high surface coverages, the entanglement of proteins plays an important role, which is not considered in our model.

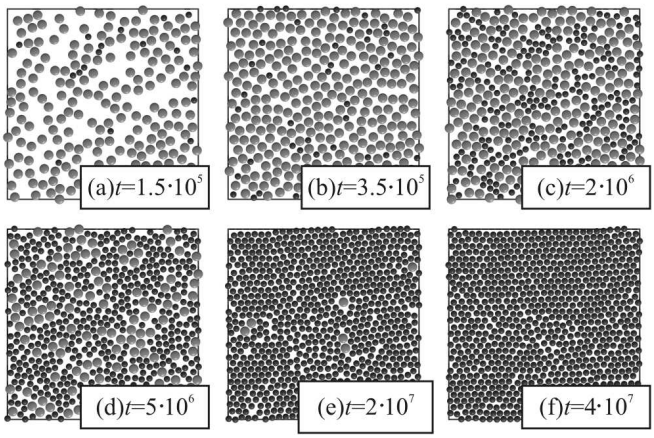

FIG. 5: Snapshots of the adsorption layer. The dark (bright) particles represent model proteins in conformation A (C).

Thus, according to the model presented, the occurrence of the discontinuity and the second linear regime in the adsorption kinetics can be ascribed to a collective transition in the internal degree of freedom of the particles, namely from a conformation that is stable on the single-particle level (C) to a conformation that optimizes adsorbed amount at the surface (A). Discrepancies of the simulated adsorption kinetics for large times are inherent to the colloidal model.

The collective transition is observed for a wide range of realistic model parameters. Whether or not the effect of surface-induced conformational changes leads to a second linear regime in the adsorption kinetics depends on how

fast the collective transition takes place. The swiftness of the transition is given by the decrease of $N_{ad,C}$ and is influenced by several simulation parameters, e.g. the particle density, the time scale of the internal degree of freedom, the ratio of the effective particle radii and the relative strength of the conformation-dependent particle-particle and particle-surface interactions.

Conclusions

In conclusion, evidence for the dependence of the adsorbed amount of protein on short-range interactions (hydrophilic/hydrophobic) as well as on the long-range van der Waals forces is demonstrated. This is contrary to the common belief that protein adsorption is predominantly determined by the short-range contribution of the surface potential.

Surprisingly, modifications of the long-range interactions result in a three-step adsorption kinetics. The simulation results indicate that this kind of kinetics originates from conformational changes of the proteins at the surface which are induced by density fluctuations.

In order to confirm the physical picture developed here, it would be ideal to perform experiments that can directly probe the conformations of the adsorbed proteins. Unfortunately, it is not possible to obtain this kind of information *in-situ* with state-of-the-art experimental methods. In addition to ellipsometry, methods are available that enable the adsorbed protein layer to be characterized in more detail, but these methods suffer from either a lack of time resolution for *in-situ* experiments (e.g. neutron scattering [27]) or from a constraint concerning the variation of substrate composition (e.g. surface plasmon resonance spectroscopy [28]). Establishing quantitative agreement between experimental and simulation results requires to refine the model approach. However, the experimental time scales and the large number of molecules restrain the complexity of a protein model, which can still be used to simulate biofilm adsorption with state-of-the-art computers. So far it is not even possible to simulate the denaturation upon adsorption of a single protein molecule in full atomistic detail, thereby gaining access to its surface native state [29]. A feasible route to improve systematically the level of sophistication of the colloidal model is to include structural information provided by coarse-grained molecular dynamics simulations [30].

Nevertheless, our observations show that long-range van der Waals forces, emananting from the substrate bulk material, may alter the properties of adsorbed protein films. Hence, for a comprehensive study of protein adsorption it is of great importance to take this type of interaction into account.

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